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This project concerns anastellin, a fragment of fibronectin that inhibits angiogenesis, tumor growth, and metastasis in vivo. It also concerns anginex, synthetic peptide with anti-angiogenic and anti-tumor effects. We have found that anginex resembles anastellin in several biochemical aspects, and that anginex, like anastellin, depends on circulating plasma fibronectin to be active in vivo. We have also found that anginex and anastellin in complex with fibronectin home to angigenic vasculature. It is possible that the fibronectin RGD sequence may function as the homing sequence of the peptide-fibronectin complexes to angiogenic endothelium in vivo.

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INTRODUCTION

Previous work in our laboratory developed anastellin, a 10 kDa fragment of the first type III repeat of fibronectin that inhibits angiogenesis, tumor growth and metastasis *in vivo* [1-3]. The structure of anastellin is a β-sheet sandwich with an exposed hydrophobic filling [4]. Anastellin polymerizes with fibronectin *in vitro* [1] and requires circulating plasma fibronectin to be anti-angiogenic *in vivo* [5]. Two other angiogenesis inhibitors, antithrombin and endostatin, also depend on fibronectin and vitronectin to be active *in vivo* [5].

Beta sheet is a common structural motif among angiogenesis inhibitors. Recently, a synthetic 33-amino acid peptide, anginex, was modeled to reproduce the β-sheet structure of anti-angiogenic proteins. Anginex inhibits angiogenesis and tumor growth [6-8], and the bioactive form of anginex has a β-sheet structure [7].

We have found that anginex, like anastellin, binds to fibronectin and initiates fibronectin polymerization. Moreover, anginex is inactive in mice that lack plasma fibronectin. We have also found that anginex- and anastellin-fibronectin complexes home to angiogenic blood vessels *in vivo*. We hypothesize that the RGD sequence may function as the homing sequence of the peptide-fibronectin complexes to angiogenic endothelium *in vivo*.

BODY

When mixed with fibronectin, anastellin induces the formation of fibronectin fibrils [1]. Because of the similarities between anastellin and anginex, we tested the effect of anginex on fibronectin. By measuring fibronectin polymerization with a turbidity assay [3, 4], we found that mixing anginex with fibronectin caused increased turbidity. Furthermore, SDS-PAGE analysis of the insoluble polymer showed that anginex co-aggregated with fibronectin (data not shown).

We tested dependency of anginex on fibronectin *in vivo* by implanting matrigel plugs into mice that lack plasma fibronectin. We found that anginex requires plasma fibronectin for its anti-angiogenic activity *in vivo* (Fig. 1). To test the hypothesis that the role of fibronectin may be to serve as a homing molecule to angiogenic endothelium *in vivo*, we allowed fluorescein-conjugated fibronectin to aggregate with anginex or anastellin, and injected the complexes into fibronectin-deficient mice carrying matrigel plugs implanted 8 days earlier. While the fluorescent fibronectin-peptide aggregates were taken up by endothelial cells in the matrigel plugs, only very few endothelial cells were found to have taken up fluorescent fibronectin alone (Fig. 2). No fluorescent aggregates were found in endothelium in control organs like the brain, heart, kidney, or pancreas. Some of the cells that had taken up the fluorescent aggregates were apoptotic (not shown). Our results suggest that fibronectin may serve as a natural guidance molecule for anginex and anastellin to angiogenic endothelium.

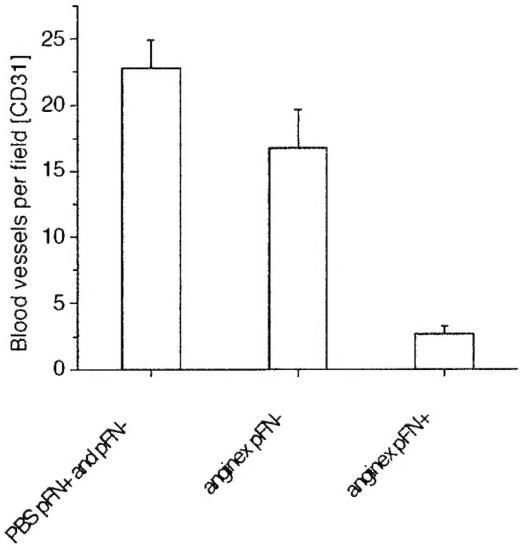

Figure 1. Anginex requires plasma fibronectin for *in vivo* activity. Mice lacking plasma fibronectin (pFN-) or their normal littermates (pFN+) were injected with matrigel impregnated with bFGF. The mice were treated with daily intraperitoneal injections of 200 μg of anginex in 0.2 ml PBS, or PBS alone, for seven days. The number of blood vessels per microscopic field (400X magnification) is shown. The data for the PBS-treated plasma fibronectin negative (pFN-) and wildtype (pFN+) mice (n = 19) were similar and have been pooled in the figure. Anginex treatment greatly reduced the blood vessel density in the pFN+ mice (n = 8), but not in the pFN- mice (n = 13). The significance level between the anginex-treated groups is $p<0.01$. The difference between the PBS-treated group and the anginex-treated pFN- groups is not significant.

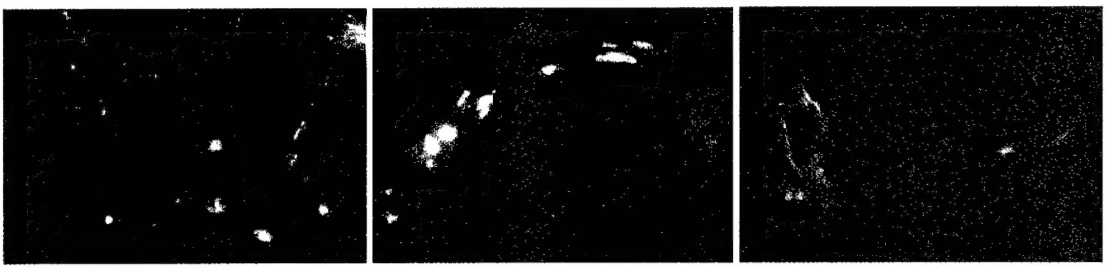

Figure 2. Anginex and anastellin in complex with fibronectin home to angiogenic vasculature *in vivo*. Fibronectin-deficient mice were injected subcutaneously with matrigel impregnated with bFGF. Eight days later, fluorescein-conjugated fibronectin (green) mixed with anastellin or anginex, or fluorescent fibronectin alone, was injected intraperitoneally and intravenously and allowed to circulate for 1 hour or 16 hours. The mice were sacrificed and the matrigel plugs removed, sectioned, and stained for blood vessels (red). Nuclei were counterstained with DAPI (blue). The injected fibronectin-peptide complexes co-localize with CD31-positive cells (anginex, **left**; anastellin, **middle**). When fluorescent fibronectin was injected alone, only a few CD31 positive cells could be found to have taken up the fluorescent fibronectin (**right**). Original magnification, 400X.

The results on the anginex/anastellin comparisons are being prepared for publication.

We also tested the effect of anastellin on lymphangiogenesis. Anastellin inhibited lymphatic vessel formation, but was not as effective in this regard as a tumor-homing

peptide isolated in our laboratory, LyP-1, which destroys tumor lymphatics and causes tumor cell apoptosis (Laakkonen et al., in press).

KEY RESEARCH ACCOMPLISHMENTS

- Anastellin is comprised of beta sheet structure.
- Like anastellin, anginex polymerizes with fibronectin *in vitro* and requires plasma fibronectin for its anti-angiogenic activity *in vivo*.
- The RGD sequence of fibronectin may serve as a homing motif to angiogenic endothelium for both anastellin and anginex *in vivo*.
- LyP-1 peptide destroys tumor lymphatics and inhibits tumor growth.

REPORTABLE OUTCOMES

- Briknarova, K., Akerman, M. E., Hoyt, D. W., Ruoslahti, E., and Ely, K. R. Anastellin, an FN3 fragment with fibronectin polymerization activity, resembles amyloid fibril precursors. *J Mol Biol* 332:205-215, 2003
- Akerman, M. E., Pilch, J., and Ruoslahti, E. The synthetic angiostatic peptide anginex requires plasma fibronectin for activity *in vivo*. Manuscript in preparation.
- Laakkonen, P., Akerman, M.E., Biliran, H., Yang, M., Ferrer, F., Karpanen, T., Hoffman, R.M., and Ruoslahti, E. Antitumor activity of a homing peptide that targets tumor lymphatics and tumor cells. *Proc. Natl. Acad. Sci. USA*. In press.

CONCLUSIONS

We have made significant progress toward understanding the mechanism of anastellin *in vivo*. The original goal of this project was to target anastellin to specific vascular sites *in vivo* by fusing anastellin with homing peptides that home to the vasculature of breast cancers. However, we have found that it is not possible to improve the efficacy of anastellin by fusing it with homing peptides. Although anastellin is thought to act on blood vessel endothelial cells, targeting anastellin to those sites by fusing it to homing peptides either inactivates anastellin, or inhibits it from acting at the site of angiogenesis. Instead, anastellin and anginex may be naturally targeted to angiogenic vasculature *in vivo* by co-aggregating with fibronectin and utilizing the RGD-sequence of fibronectin to home to angiogenic vasculature.

REFERENCES

1. Morla, A., Z. Zhang, and E. Ruoslahti. *Nature*, 1994. 367(6459): p. 193-6.
2. Pasqualini, R., *et al. Nat Med*, 1996. 2(11): p. 1197-203.
3. Yi, M. and E. Ruoslahti. *Proc Natl Acad Sci U S A*, 2001. 98(2): p. 620-4.
4. Briknarova, K., *et al. J Mol Biol*, 2003. 332(1): p. 205-15.
5. Yi, M., *et al. Proc Natl Acad Sci U S A*, 2003. 100(20): p. 11435-8.
6. Griffioen, A.W., *et al. Biochem J*, 2001. 354(Pt 2): p. 233-42.
7. Dings, R.P., *et al. Biochem J*, 2003. 373(Pt 1): p. 281-8.
8. van der Schaft, D.W., *et al. Faseb J*, 2002. 16(14): p. 1991-3.